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(54) Title: DETERGENT ENZYMES

(57) Abstract

This invention is in the field of detergent enzymes. More specifically, the invention relates to the use of proteases derived from fungi of the genus *Verticillium* for detergent purposes.

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DETERGENT ENZYMES

TECHNICAL FIELD

This invention is in the field of detergent enzymes. More specifically, the invention relates to the use of proteases derived from fungi of the genus Verticillium for detergent purposes.

BACKGROUND ART

Fungi belonging to the genus Verticillium are well known in the literature, and they are known to be pathogenic to insects and plants. The fungi are also known to produce proteolytic enzymes which have been investigated in relation to the pathogenicity of the fungi.

SUMMARY OF THE INVENTION

It has now surprisingly been found that proteases derived from members of the fungi Verticillium posses excellent washing performance.

Accordingly, the present invention provides detergent compositions comprising proteases obtainable from, or proteases having immunochemical properties identical or partially identical to those of a protease derived from any of the strains CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; and CBS No. 464.88.

In another aspect, the invention provides detergent compositions comprising proteases obtainable from a member of the genus Verticillium, or a mutant or a variant thereof.

In yet another aspect, the invention provides detergent additives comprising proteases obtainable from, or proteases having immunochemical properties identical or partially identical to those of a protease derived from any of the strains CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; and CBS No. 464.88.

In a further aspect, the invention provides detergent additives comprising proteases obtainable from a member of the genus Verticillium, or a mutant or a variant thereof.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows the relation between temperature (°C) and proteolytic activity (% relative) of an enzyme of the invention (■ at pH 9.5; □ at pH 9.5 with 0.1% STPP added); and

Fig. 2 shows the relation between pH and proteolytic activity (% relative) of an enzyme of the invention.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides detergent compositions comprising proteases obtainable from a fungal strain of the genus Verticillium. Fungi belonging to the genus Verticillium are well known and described in the literature. Strains of Verticillium have been deposited and made available from various international depositary institutes, e.g. CBS No. 247.68; CBS No. 145.70; CBS No. 146.70; or CBS No. 464.88.

The proteases are obtainable by methods known and described in the literature, e.g. by cultivation of a protease producing strain of the genus Verticillium in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the desired enzyme, or may e.g. be produced by employing recombinant DNA technology.

The Proteases

In the context of this invention, suitable proteases are the proteases obtainable from strains of Verticillium, or mutants or variants thereof, or proteases

having immunochemical properties identical or partially identical to a protease obtainable from a strain of Verticillium, e.g. V. bulbillosum.

By an enzyme variant or mutated enzyme is meant an enzyme obtainable by alteration of the DNA nucleotide sequence of the parent gene or its derivatives. The enzyme variant or mutated enzyme may be expressed and produced when the DNA nucleotide sequence encoding the enzyme is inserted into a suitable vector in a suitable host organism. The host organism is not necessarily identical to the organism from which the parent gene originated.

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to N. H. Axelsen; Handbook of Immuno-precipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, chapters 5, 19 and 20.

Detergent Compositions

The detergent composition of the invention may comprise one or more surfactants, which may be of an anionic, non-ionic, cat-ionic, amphoteric or zwitter-ionic type, or a mixture of these. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS); alkyl sulfates (AS); alpha olefin sulfonates (AOS); alcohol ethoxy sulfates (AES) and alkali metal salts of natural fatty acids. Examples of non-ionic surfactants are alkyl polyethylene glycol ethers; nonylphenol polyethylene glycol ethers; fatty acids esters of sucrose and glucose; and esters of polyethoxylated alkyl glucoside.

The detergent composition of the invention may also contain other detergent ingredients known in the art such as builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, stabilizers for the enzymes and bleaching agents, formulations aids, optical brighteners, foam boosters, chelating agents, fillers, fabric softeners, etc. The detergent composition of the invention may be formulated substantially as described

in J. Falbe [Falbe, J.; Surfactants in Consumer Products. Theory, Technology and Application; Springer Verlag 1987, vide in particular the section entitled "Frame formulations for liquid/powder heavy-duty detergents"].

It is at present contemplated that the detergent composition of the invention may contain the enzyme preparation in an amount corresponding to 0.0005-0.5 CPU of the proteolytic enzyme per litre of washing liquor.

The detergent compositions of the invention can be formulated in any convenient form, such as powders, liquids, etc.

The detergent composition of the invention may advantageously include one or more other enzymes, e.g. lipases; amylases; cellulases; oxidases; and/or peroxidases, conventionally included in detergent compositions, as well as proteases of other origin.

The protease of the invention may be included in a detergent composition by adding separate additives containing the detergent protease, or by adding a combined additive comprising different detergent enzymes.

The additive of the invention, i.e. a separated additive or a combined additive, can be formulated e.g. as granulates, liquids, slurries, etc. Preferred detergent additive formulations are non-dusting granulates, liquids, in particular stabilized liquids, slurries, or protected enzymes. Dust free granulates may be produced according to e.g. GB Patent No. 1,362,365 or US Patent No. 4,106,991, and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as e.g. propylene glycol; a sugar or sugar alcohol; lactic acid or boric acid, according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP Patent Application No. 238,216.

The invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

Preparation Example

The strain Verticillium bulbillosum, CBS 247.68, was cultivated at 25°C on a rotary shaking table (240 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of medium of the following composition (per litre):

Sucrose	100 g
Soybean flour	40 g
$Na_2HPO_4 \times 12\ H_2O$	10 g
Pluronic®	0.1 g

The medium is sterilized by heating at 120°C for 45 minutes.

After 12 days of incubation a proteolytic activity of the culture of 21 CPU/l was determined using the method described below.

After separation of the solid material the protease was purified by a conventional chromatographic method.

Yield from 1 l of culture broth was 30 ml with 270 CPU/l. Purity was more than 90% as judged by SDS-PAGE.

Preparations from the strains V. bulbillosum, CBS 145.70; V. bulbillosum, CBS 146.70; and V. suchlasporium var. suchlasporium, CBS 464.88, were obtained in similar ways.

EXAMPLE 2

Characterization of the Enzyme

The preparation prepared in accordance with Example 1 was subjected to the following characterization.

A molecular weight of 30 kD was determined by SDS-PAGE. A pI higher than 9.3 was determined by isoelectric focusing on LKB Ampholine® PAG plates. The protease activity is inhibited by PMSF, α-1-antitrypsin and Turkey-egg-

white proteinase inhibitor. EDTA and soybean-protein inhibitor do not influence the protease activity.

The temperature activity relationship was determined with casein as substrate. The assay for proteolytic activity described previously was used with the modification that the incubation temperature was varied in the interval of from 15 to 70°C. The result is shown in Fig. 1. The enzyme possesses proteolytic activity from temperatures below 15°C to above 70°C, and a temperature optimum within the range of 45 to 65°C; around 55°C.

The dependence of activity on pH was determined by the same procedure, using buffers adjusted to predetermined pH values in the pH range of from 6 to 11. The result is shown in Fig. 2. The enzyme possesses proteolytic activity at pH values below 6 to above 11, with a pH optimum in the range of pH 8 to pH 11.

Assay for Proteolytic Activity

The proteolytic activity is determined with casein as substrate. One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1 mM of primary amino groups (determined by comparison with a serine standard) per minute under standard conditions, i.e. incubation for 30 minutes at 25°C and pH 9.5. A folder AF 228, describing the analytical method, is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

EXAMPLE 3

Wash Performance

Two sets of wash performance tests were accomplished on grass juice soiled cotton at 20°C, isothermally for 10 minutes.

In the first set 2.0 g/l of a commercial American type powder detergent were used. The detergent was dissolved in approx. 6° dH (German Hardness) water. The pH was 9.5. The results of these tests are shown in Table 1.

In the second set 2.0 g/l of a commercial American type powder detergent with bleach and activator were used. The detergent was dissolved in approx. 6° dH (German Hardness) water. The pH was 9.5. The results of these tests are shown in Table 2.

In both sets the textile/wash liquor ratio was 6 g of textile per litre of wash liquor.

Subsequent to washing, the cloths were rinsed in running tap-water and air-dried. The remission (%R) was determined at 460 nm.

As a measure of the wash performance differential remission, ΔR, was used being equal to the remission after wash with enzyme added, minus the remission after wash with no enzyme added.

Table 1

The differential remission, Δ R, measured after wash in a commercial American type powder detergent.

Strain No. (CBS)	Enzyme dosage (CPU/l) 0.01	0.05	0.1	0.5
145.70	11	17.8	18.9	19.3
247.68	7.6	16	19	19.7
464.88	8.1	15.2	19.3	19.9

Table 2

The differential remission, Δ R, measured after wash in a commercial American type powder detergent with bleach and activator.

Strain No. (CBS)	Enzyme dosage (CPU/l) 0.01	0.05	0.1	0.5
145.70	8.4	13.5	13.6	13.8
247.68	8.4	12.8	14.1	14.1
464.88	7.3	13.0	14.4	14.7

As indicated by the differential remission values the proteases of the invention are well suited for use as detergent enzymes.

CLAIMS

1. A detergent composition comprising one or more proteases obtainable from, or one or more proteases having immunochemical properties identical or partially identical to those of a protease derived from any of the strains CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; and CBS No. 464.88.

2. A detergent composition comprising one or more proteases obtainable from a member of the genus Verticillium, or a mutant or a variant thereof.

3. The detergent composition of claim 2, comprising one or more proteases obtainable from the strain CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; or CBS No. 464.88, or a mutant or a variant thereof.

4. The detergent composition of claim 2, comprising one or more proteases obtainable from the strain CBS No. 145.70; CBS No. 146.70; or CBS No. 247.68, or a mutant or a variant thereof.

5. A detergent composition of any of claims 1-4, which composition further comprises one or more other enzymes, in particular amylases, lipases, cellulases, oxodases, and/or peroxidases.

6. A detergent additive comprising one or more proteases obtainable from, or one or more proteases having immunochemical properties identical or partially identical to those of a protease derived from any of the strains CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; and CBS No. 464.88.

7. A detergent additive comprising one or more proteases obtainable from a member of the genus Verticillium, or a mutant or a variant thereof.

8. The detergent additive of claim 7, comprising one or more proteases obtainable from the strain CBS No. 145.70; CBS No. 146.70; CBS No. 247.68; or CBS No. 464.88, or a mutant or a variant thereof.

9. The detergent additive of claim 7, comprising one or more proteases obtainable from the strain CBS No. 145.70; CBS No. 146.70; or CBS No. 247.68, or a mutant or a variant thereof.

10. A detergent additive according to either of claims 6-9, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

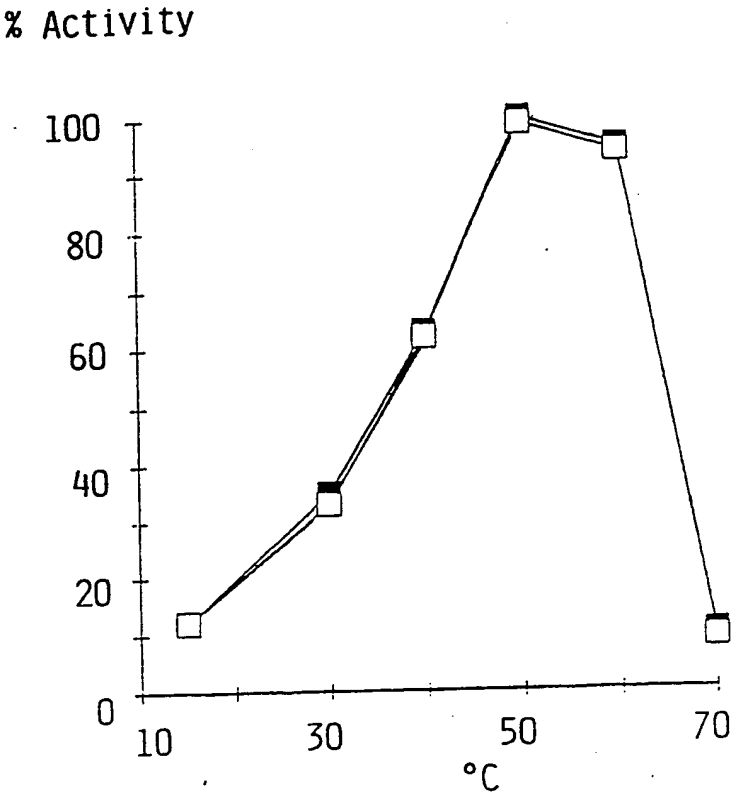

Fig. 1

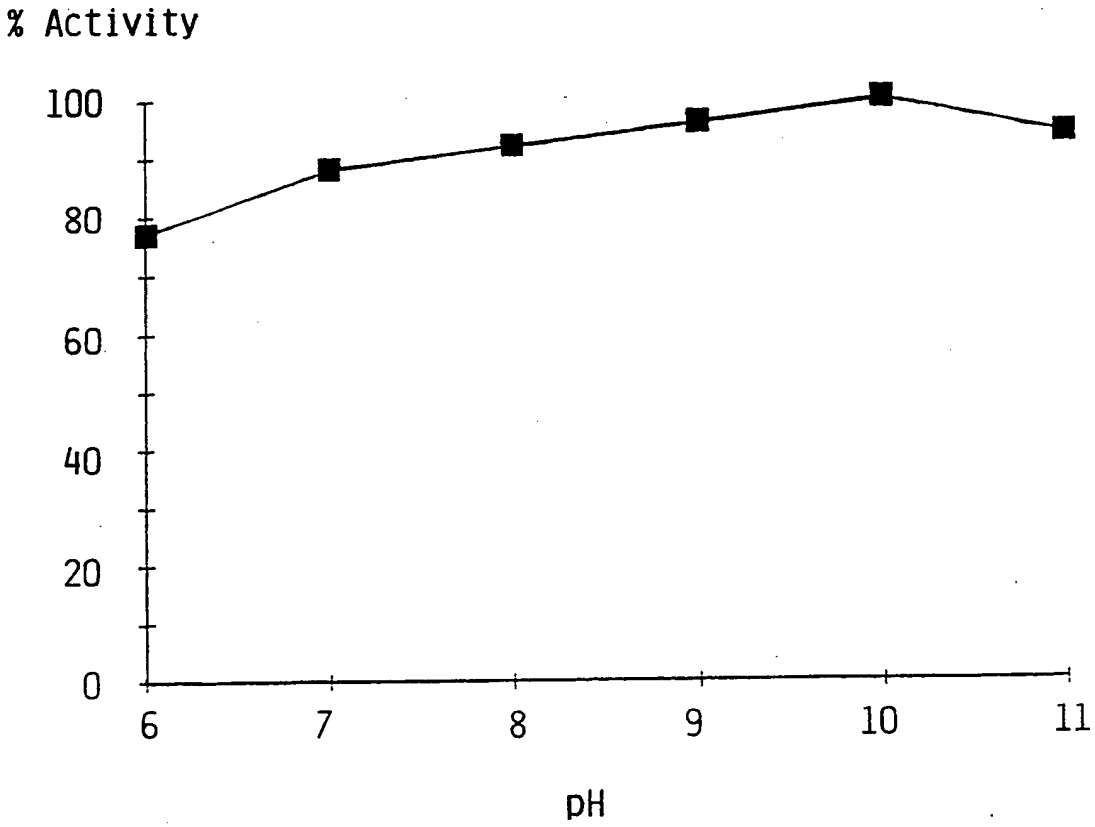

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00273

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 11 D 3/386, C 12 N 9/58

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 11 D; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP, A1, 0335023 (AGRICULTURAL GENETICS COMPANY LIMITED) 4 October 1989, see the whole document --	1-10

* Special categories of cited documents: [10]

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"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th December 1992	21-12-1992
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Dagmar Järvman

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 92/00273

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 02/12/92
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0335023	89-10-04	US-A- 4987077	91-01-22